Macrophage-Derived IL-33 Is a Critical Factor for Placental Growth

Valerie Fock, Mario Mairhofer, Gerlinde R. Otti, Ursula Hiden, Andreas Spittler, Harald Zeisler, Christian Fiala, Martin Knöfler and Jürgen Pollheimer

*J Immunol* 2013; 191:3734-3743; Prepublished online 30 August 2013;
doi: 10.4049/jimmunol.1300490
http://www.jimmunol.org/content/191/7/3734

Supplementary Material  
http://www.jimmunol.org/content/suppl/2013/08/30/jimmunol.1300490.DC1

References  
This article cites 61 articles, 22 of which you can access for free at:  
http://www.jimmunol.org/content/191/7/3734.full#ref-list-1

Subscription  
Information about subscribing to *The Journal of Immunology* is online at:  
http://jimmunol.org/subscription

Permissions  
Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  
Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts
Macrophage-Derived IL-33 Is a Critical Factor for Placental Growth

Valerie Fock,* Mario Mairhofer,1 Gerlinde R. Otti,‡ Ursula Hiden,§ Andreas Spittler,§ Harald Zeisler,⁎ Christian Fiala,§ Martin Knöfler,⁎ and Jürgen Pollheimer*“

IL-33, the most recently discovered member of the IL-1 superfamily and ligand for the transmembrane form of ST2 (ST2L), has been linked to several human pathologies including rheumatoid arthritis, asthma, and cardiovascular disease. Deregulated levels of soluble ST2, the natural IL-33 inhibitor, have been reported in sera of preeclamptic patients. However, the role of IL-33 during healthy pregnancy remains elusive. In the current study, IL-33 was detected in the culture supernatants of human placental and decidual macrophages, identifying them as a major source of secreted IL-33 in the uteroplacental unit. Because flow cytometry and immunofluorescence staining revealed membranous ST2L expression on specific trophoblast populations, we hypothesized that IL-33 stimulates trophoblasts in a paracrine manner. Indeed, BrdU incorporation assays revealed that recombinant human IL-33 significantly increased proliferation of primary trophoblasts as well as of villous cytotrophoblasts and cell column trophoblasts in placental explant cultures. These effects were fully abolished upon addition of soluble ST2. Interestingly, Western blot and immunofluorescence analyses demonstrated that IL-33 activates AKT and ERK1/2 in primary trophoblasts and placental explants. Inhibitors against PI3K (LY294002) and MEK1/2 (UO126) efficiently blocked IL-33–induced proliferation in all model systems used. In summary, with IL-33, we define for the first time, to our knowledge, a macrophage-derived regulator of placental growth during early pregnancy. The Journal of Immunology, 2013, 191: 3734–3743.

Proper development of the placenta is pivotal for a successful pregnancy as placental trophoblasts are required for hormone secretion, blood supply and immunological acceptance of the allogeneic embryo. The basic structural units of the placenta are placental villi, which are composed of a stromal villous core (VC) and surrounded by a layer of epithelial villous cytotrophoblasts (vCTBs), harboring a putative trophoblast stem cell niche (1). The VC contains fetal macrophages (also known as Hofbauer cells), blood vessels and fibroblasts. During early pregnancy, vCTBs either fuse to form multinucleated syncytiotrophoblasts (STs) or differentiate into extravillous trophoblasts (EVTs) (2), see also Fig. 7A). STs cover placental villi and fulfill substantial functions during pregnancy, such as nutrient and metabolite transport or hormone production (3, 4). Determination of the EVT lineage starts with the formation of proliferative cell column trophoblasts (CCTs), which upon growth arrest differentiate into highly invasive EVTs (also referred to as interstitial cytotrophoblasts). The latter deeply invade the deciduized endometrium and the first third of the myometrium and participate in the remodeling of spiral arteries to increase the blood flow to the developing fetus (5).

Regulatory interactions between vCTBs and underlying cells of the placental mesenchymal VC as well as between the EVT lineage and decidual cells are believed to be influential factors of adequate trophoblast function. In this context, there is growing evidence for the involvement of uteroplacental (placental and decidual) leukocyte populations in the paracrine stimulation of various trophoblast subtypes. Although macrophages represent the only immune cell population in the placental VC, leukocytes in the decidua mainly consist of macrophages (20–30%) (6) and uterine NK (uNK) cells (60–70%) (7). Notably, the secretome of Hofbauer cells (from now on referred to as placental macrophages, pMφs) was described to influence proliferation and function of vCTBs (8, 9), whereas decidual macrophages (dMφs) secrete factors known to alter trophoblast motility (10, 11). aberrant activation of placental or decidual leukocytes seems integral in the development of various pregnancy-related disorders, because spontaneous abortions and preeclampsia (PE) are accompanied by deregulated uteroplacental and/or serum levels of pro- and anti-inflammatory cytokines including IFN-γ, TNF, and IL-10 (12-15). Interestingly, soluble ST2 (sST2), the natural inhibitor of the IL-1 family member IL-33 (16), was recently reported to be upregulated in the serum of preeclamptic women (17). The ligand for the transmembrane form of ST2 (ST2L) is activated upon binding of IL-33, which leads to heterodimerization with IL-1R accessory protein and cell type–dependent activation of NF-κB, PI3K/AKT, and MAPKs (16, 18, 19). IL-33 can act as full-length 31-kDa protein (20) or cleaved fragment (21) and was initially described to induce Th2-related immune responses (16). Besides its versatile effects on various leukocyte populations, IL-33 also promotes inflammatory activation,

*Reproductive Biology Unit, Department of Obstetrics and Fetal–Maternal Medicine, Medical University of Vienna, 1090 Vienna, Austria; †Department of Gynecological Endocrinology and Reproductive Medicine, Medical University of Vienna, 1090 Vienna, Austria; ‡Department of Obstetrics and Gynecology, Medical University of Graz, 8036 Graz, Austria; §Core Facility Flow Cytometry and Surgical Research Laboratories, Medical University of Vienna, 1090 Vienna, Austria; and Gymnmed Clinic, Vienna, 1090 Vienna, Austria

Received for publication February 20, 2013. Accepted for publication July 25, 2013.

This work was supported by “Jubiläumsfonds” of the Austrian National Bank Grants 13955 (to H.Z.) and 14147 (to J.P.) and Austrian Science Fund Grants P-25187-B13 (to J.P.) and P-25187-B13 (to J.P.).

Address correspondence and reprint requests to Dr. Jürgen Pollheimer, Reproductive Biology Unit, Department of Obstetrics and Fetal–Maternal Medicine, Medical University of Vienna Währinger Gürtel 18-20, 1090 Vienna, Austria. E-mail address: juergen.pollheimer@meduniwien.ac.at

The online version of this article contains supplemental material.

Abbreviations used in this article: CCT, cell column trophoblast; dMφ, decidual macrophage; EC, endothelial cell; EVT, extravillous trophoblast; HAI1, hepatocyte growth factor activator inhibitor type 1; HPAEC, human placental arterial endothelial cell; IGF, insulin-like growth factor; KRT, keratin; KRT7, cytokeratin-7; PE, preeclampsia; pMφ, placental macrophage; rlh, recombinant human; sST2, soluble ST2; ST, syncytiotrophoblast; ST2L, ligand for the transmembrane form of ST2; uNK cell, uterine NK cell; VC, villous core; vCTB, villous cytotrophoblast.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/$16.00
proliferation, and motility of endothelial cells (ECs) (18, 19) as well as secretion of immunomodulatory factors from fibroblasts (22) and epithelial cells (23). Nuclear expression of IL-33 in ECs has been linked to transcriptional activity (24) and passive leakage upon necrosis (20), whereas its active secretion has been demonstrated from fibroblasts (25), epithelial cells (26, 27), and macrophages (28) in mice.

Cytokines have been shown to exert profound effects on trophoblasts in vitro and thus likely contribute to placental development. Hence, elevated sST2 serum levels during PE raise the question whether its target IL-33 could influence trophoblast function because this pathological condition is of placental origin. However, the role of IL-33 during early placental development has not yet been elucidated and was therefore scope of this study.

Materials and Methods

Tissue collection
Placental (n = 70) and decidual (n = 20) specimens were obtained from elective terminations of viable first-trimester pregnancies (6–12th gestational week). The gestational age was determined by ultrasound. Patients were locally anesthetized and treated with misoprostol before surgical intervention. Tissues were collected with informed consent, and utilization was approved by the Ethics Committee of the Medical University of Vienna. Human term placentae of healthy pregnancies were collected after vaginal delivery under informed consent. Approval was granted by the Ethics Committee of the Medical University of Graz.

Isolation of primary first-trimester human trophoblasts and macrophages
Trophoblasts were isolated according to a modified protocol of Tarrade et al. (29). Briefly, placental villi were scraped and digested in 0.125% trypsin (Life Technologies, Carlsbad, CA) and 12.5 mg/ml DNase I (Sigma-Aldrich, St. Louis, MO) in 10% Ca2+/Mg2+-free HBSS (Sigma-Aldrich) for 30 min at 37˚C. Cell suspensions were filtered through a 70-µm cell strainer (BD Biosciences, Franklin Lakes, NJ) and separated by Percoll density gradient centrifugation (Pharmacia, Uppsala, Sweden). Cells between 35 and 50% of the Percoll layer were collected and incubated in RBC lysis buffer (155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA [pH 7.3]) for 5 min at room temperature. Cells were taken up in DMEM/Ham’s F-12 medium (PAA Laboratories), 0.05 mg/ml gentamicin, and 0.5 µg/ml fungizone (both from Life Technologies), and plated on plastic dishes for 40 min to remove contaminating fibroblasts. Finally, trophoblasts were collected and seeded onto fibronectin-coated (20 µg/ml; BD Biosciences) wells at a density of 2.5 × 10⁴ cells/well. Macrophages were obtained from placental and decidual isolates by positive selection with anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultivated in DMEM/Ham’s F-12 medium, supplemented with 10% FCS and 0.05 mg/ml gentamicin in the absence or presence of recombinant human (rhu) IL-1β (10 ng/ml).

Isolation of primary first-trimester human decidual stromal cells
Decidual stromal cells were isolated as described previously (30). Briefly, decidual tissue was minced into 3-mm³ pieces and digested under agitation in 2 mg/ml collagenase I (Life Technologies) and 0.5 mg/ml DNase I in HBSS containing 25 mM HEPES (Life Technologies) for 1 h at 37°C. Dispersed cells were pooled and filtered through a 70-µm cell strainer. After RBC lysis, cells were processed for cell sorting.

Isolation of primary third-trimester human placental arterial endothelial cells
Human placental arterial endothelial cells (HPAECs) were isolated from human term placenta as described previously (31). Briefly, arterial blood vessels were resected from the apical surface of the chorionic plate and washed with HBSS to remove residual blood. HPAECs were isolated by perfusion of choriocarcin arteries with 0.1 U/ml collagenase and 0.8 U/ml dispase (Roche Diagnostics, Mannheim, Germany) in HBSS for 7 min. Cells were resuspended in endothelial basal medium (Lonza, Verviers, Belgium), supplemented with 5% FCS, and cultivated on 1% gelatin-coated (Sigma-Aldrich) plates.

First-trimester placental explant cultures
To evaluate trophoblast outgrowth from placental explant cultures, placental villi (7–9th gestational week) were dissected and cultivated on collagen I (BD Biosciences) (32) in the absence or presence of rhu IL-33 (10 or 100 ng/ml; Enzo Life Sciences, Lause, Switzerland) or 0.1% BSA (vehicle).
The area of outgrowth was digitally photographed (Olympus IX71, ColorView III digital camera, Cell*ID software; Olympus, Hamburg, Germany) after 0, 24, 48, and 72 h of cultivation.

Flow cytometry and FACS

Single-cell suspensions of overnight-cultivated trophoblasts were obtained by EDTA treatment and scraping. Cells were resuspended in ice-cold PBS with 2% BSA and stained with an anti-ST2 Ab for 30 min at 4°C. An appropriate isotype-specific control Ab was used accordingly. Data were acquired on a FACScan (BD Biosciences) flow cytometer and analyzed with FlowJo 7.6.4 software (Tree Star, Ashland, OR). For cell sorting of placental and decidual leukocytes, cell isolates were labeled with an anti-CD56 Ab. To obtain uNK cells, decidual isolates were cultured for 24 h in RPMI 1640 medium, supplemented with 10% FCS and 0.05 mg/ml gentamicin to reduce CD56 expression, and subsequently, nonattached cells were stained with an anti-CD56 Ab. Sorting was performed on a MoFlo Astrios cell sorter (Beckman Coulter).

Immunofluorescence stainings

First-trimester placental and decidual tissue samples or placental floating explants were fixed with 7.5% formaldehyde and embedded in paraffin (Merck, Darmstadt, Germany). Deparaffinized tissue sections (3 μm) were boiled in 1× Target Retrieval Solution (pH 6.1) (DakoCytomation, Glostrup, Denmark) and incubated with 0.05% fish skin gelatin (Sigma-Aldrich), followed by incubation with primary Abs outlined in Supplemental Table I overnight at 4°C. Appropriate isotype-specific control Abs were used accordingly. Subsequently, sections were incubated with goat anti-mouse or anti-rabbit IgG conjugated to Alexa Fluor 488 or Alexa Fluor 568 (2 μg/ml; Molecular Probes, Life Technologies) for 1 h at room temperature, counterstained with DAPI (1 mg/ml; Roche Diagnostics), and mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Images were acquired on a fluorescence microscope (Olympus BX50, CC12 digital camera, Cell*ID software; Olympus).

PI3K/AKT and MAPK/ERK signaling in primary trophoblasts

To investigate activation of signaling pathways, overnight-cultivated primary trophoblasts were serum-starved for 4 h, followed by preincubation with rhu sST2 (1 μg/ml; R&D Systems, Minneapolis, MN), LY294002, or UO126 (10 μM each; Cell Signaling Technology, Danvers, MA) for 1 h. Subsequently, rhu IL-33 (100 ng/ml) or 0.1% BSA (vehicle) were added to the cells, which were further maintained for 30 min before being processed for Western blotting.

Western blot analysis

Cells were lysed in Laemmli buffer, followed by boiling for 10 min. Proteins were separated by SDS-PAGE and blotted onto methanol-activated poly(vinylidene difluoride) membranes (GE Healthcare, Buckinghamshire, U.K.). After blocking in 5% nonfat dry milk in TBST, membranes were incubated with primary Abs outlined in Supplemental Table I overnight at 4°C, followed by incubation with HRP-linked anti-mouse or anti-rabbit IgG conjugated to Alexa Fluor 488 or Alexa Fluor 568 (2 μg/ml; Molecular Probes, Life Technologies) for 1 h at room temperature, counterstained with DAPI (1 mg/ml; Roche Diagnostics), and mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Images were acquired on a fluorescence microscope (Olympus BX50, CC12 digital camera, Cell*ID software; Olympus).

Proliferative and invasive trophoblasts express ST2L. (A) Immunofluorescence costainings of ST2 and the proliferation marker Ki67 or View III digital camera, Cell^D software; Olympus, Hamburg, Germany) after 0, 24, 48, and 72 h of cultivation.

Invasion assay

Invasion of 48 h cultivated growth-arrested trophoblasts was studied by using Transwell inserts with a pore size of 12 μm (Merck Millipore, Billerica, MA). Inserts were coated with 0.5 mg/ml fibronectin and placed into 24-wells containing 300 μl DMEM/Ham’s F-12 medium supplemented with 10% FCS and 0.05 mg/ml gentamicin. Subsequently, 1×10⁵ primary trophoblasts were resuspended in 400 μl DMEM/Ham’s F-12 medium supplemented with 0.1% FCS and added to the upper chamber. Each invasion condition was performed in the absence or presence of rhu IL-33 (10 or 100 ng/ml) and/or rhu ST2 (1 μg/ml). A total of 0.1% BSA was used as vehicle control. After 48 h of incubation at 37°C, inserts were fixed with ice-cold methanol, and noninvaded cells were removed using a cotton swab. Invaded cells were stained with an anti-cyto-keratin-7 (KRT7) Ab and counterstained with DAPI. Finally, inserts were embedded in Fluoromount-G and analyzed by fluorescence microscopy.

Proliferation assay

Proliferation of primary trophoblasts was assessed by using BrdU Labeling and Detection Kit II (Roche Diagnostics). Briefly, freshly isolated trophoblasts were preincubated with rhu sST2 (1 μg/ml), LY294002, or UO126 (10 μM each) for 1 h, followed by addition of rhu IL-33 (100 ng/ml) or 0.1% BSA (vehicle) for 18 h. Finally, BrdU (10 μM) was added for the last 2 h of culture. After fixation, cells were stained with anti-BrdU and anticytokeratin wide spectrum (KRT) Abs and counterstained with DAPI. Placental floating explants were cultivated in DMEM/Ham’s F-12 medium containing 0.05 mg/ml gentamicin and 10 μM BrdU. After preincubation with rhu sST2 (1 μg/ml), LY294002 or UO126 (10 μM each) for 1 h, rhu IL-33 (100 ng/ml), or 0.1% BSA was added to the cultures, which were further maintained for 18 h before being processed for immunofluorescence stainings.

ELISA

IL-33 levels in culture supernatants of pMøs, dMøs, HPAECs, and HUVECs were determined using a sandwich-type ELISA (Legend Max; BioLegend, San Diego, CA), according to the manufacturer’s instructions. Results were derived using the standard curve method.
**Statistical analysis**

Statistical analysis was performed with Student t test, p < 0.05 were considered significant. Gaussian distribution and equality of variances were examined with Kolmogorov–Smirnov test and Levene’s test, respectively, using SPSS version 18 (SPSS).

**Results**

**IL-33 is expressed by CD14+ macrophages in the uteroplacental unit**

IL-33 expression has been noticed in tissue-resident epithelial cells and ECs as well as in cells of the hematopoietic lineage (16). To identify IL-33–expressing cells in first-trimester placental and decidual tissue, respective cell isolates were therefore sorted into CD45- and CD45+ populations. Subsequent Western blot analyses revealed IL-33 expression in CD45+ cells originating from both placenta and decidua. IL-33 was found to be expressed as two immunoreactive bands, the full-length 31-kDa protein, and a shorter fragment of ~18–20 kDa (Fig. 1A). These data suggest leukocytes as a major source for IL-33 in the uteroplacental unit. In line with these results, immunofluorescence stainings of placental and decidual tissue sections showed cytoplasmic IL-33 expression in CD45+ cells (Supplemental Fig. 1A). To define the leukocyte subset expressing IL-33, we isolated CD14+ pMφs and dMφs. The purity of macrophage cultures was verified by expression of CD45 and the macrophage marker CD68 (Supplemental Fig. 3). In addition, we sorted CD56+ uNK cells by flow cytometry. Western blot analyses showed strong IL-33 expression in both macrophage populations, but the respective cytokine was not detected in uNK cells (Fig. 1B). Immunofluorescence costainings of IL-33 and CD45 confirmed these findings, showing strong cytoplasmic localization of IL-33 in CD14+ pMφs and dMφs (Fig. 1C). Because there is recent evidence for the existence of a CD14+CD68+ side population in the decidua (33), we performed costainings of IL-33 with CD68, verifying that CD68+ dMφs also express IL-33 (Supplemental Fig. 1B). CD56+ uNK cells were negative for IL-33 (data not shown). In addition, we detected a prominent nuclear staining of IL-33 in ECs located in the placental VC and in the decidua (Supplemental Fig. 2A), supporting previous findings in other organs (34). IL-33 was not detectable in KRT+ trophoblasts or vimentin+ stromal cells of placental and decidual tissue sections (Supplemental Fig. 1C). Immunofluorescence stainings of third-trimester placental and decidual tissue showed that macrophages maintain cytoplasmic IL-33 expression until term of pregnancy (Supplemental Fig. 2B). Finally, ELISA of pMφ- and dMφ-derived culture supernatants revealed secretion of ~400 pg/ml IL-33, which was further enhanced in the presence of rhu IL-1β (Fig. 1D). Although existing literature suggests that IL-33 is not secreted by viable ECs (20, 35, see also Discussion), we assessed IL-33 secretion from third-trimester HPAECs and HUVECs. In addition, supernatants of cultivated placental villous fibroblasts and decidual stromal cells were analyzed. ELISA confirmed that the respective cells do not secrete IL-33 (Fig. 1D; data not shown).

**ST2L is expressed by various trophoblast subtypes**

To determine IL-33 target cells in the uteroplacental unit, we performed immunofluorescence stainings of first-trimester placental and decidual tissue sections using specific Abs against ST2, recognizing both ST2L and sST2 (Fig. 2A; data not shown). Interestingly, proliferating KI67+ vCTBs and CCTs as well as invasive HLA-G1+ EVTs in the decidua-stained positive for the IL-33R. In addition, we observed occasional ST2 expression in vimentin+ decidual stromal cells (data not shown). To test whether isolated primary trophoblasts maintain ST2L expression in vitro over time, we performed Western blot and flow cytometric analyses of differentiating trophoblast cultures. When cultivated on extracellular matrices, these cells gradually differentiate into invasive EVTs (32, 36, 37) as demonstrated by the induction of integrin α5 (Fig. 2B), a well-accepted marker for the respective trophoblast lineage (38). Western blot analyses revealed stable expression of ST2L in both early and late trophoblast cultures, the latter expressing considerably higher levels of the natural IL-33 inhibitor sST2 (Fig. 2B). Finally, flow cytometric analyses showed that cultivated trophoblasts express membranous ST2L (Fig. 2C).

**FIGURE 3.** EVT formation in placental explants is enhanced by rhu IL-33. (A) First trimester placental explants (7–9th week) were cultivated on collagen I up to 72 h in the absence or presence of rhu IL-33 (10 or 100 ng/ml) or rhu IGF2 (100 ng/ml). Displacement of cells was measured as the length (millimeters) between the villous tip (starting point of EVT formation) and migratory front (final position of EVTs) using Photoshop CS5 (Adobe). Data from four independent experiments are shown as percentage of the mean (± SD). #, IL-33 treatment (100 ng/ml) compared with vehicle-treated control at the same time point; *, respective treatments compared with unstimulated control at day 0 (set to 100%). Representative photographs of placental explants cultivated in the absence or presence of rhu IL-33 (100 ng/ml) for 48 h are shown. Migratory front and villous tips are displayed by a white dotted line; length of cellular displacement is shown by arrows. Scale bars, 50 μm. (B) Primary trophoblasts were cultivated on fibronectin for 48 h to induce growth arrest, followed by incubation on fibronectin-coated Transwells (12 μM pore size) in the absence or presence of rhu IL-33 and/or sST2 at the indicated concentrations for another 48 h. Five nonoverlapping pictures of each insert representing ~50% of the overall surface were taken at a 100-fold magnification and cells were digitally counted using ImageJ 1.45 software. Invasion was evaluated as the percentage of invaded KRT7+ cells relative to vehicle-stimulated control (100%). Bars represent the percentage of the mean (± SD) of three independent experiments performed in duplicates (**p < 0.05).
Altogether, these data suggest that trophoblasts constitute the major target of secreted IL-33 in the uteroplacental unit.

**IL-33 induces EVT formation in first trimester placental explant cultures**

Because the commonly used 18-kDa fragment of IL-33 was recently shown to be nonexistent in humans (21, 39, 40), we used rhu full-length IL-33, which activates ST2L with similar kinetics (20). First, we assessed the effect of rhu IL-33 on first-trimester placental explants mimicking EVT differentiation by the formation of proliferative CCTs, which differentiate into growth-arrested migratory EVTs (41). The extent of EVT formation was quantified as the length of trophoblast displacement from the villous tip to the migratory front. Interestingly, IL-33 treatment significantly enhanced EVT formation when compared with vehicle-stimulated control measured at 24, 48, and 72 h (Fig. 3A). The total extent of EVT formation in control-treated explant cultures reached a 3.8-fold gain at day 3, whereas addition of 100 ng/ml IL-33 provoked a 5-fold increase when compared with respective untreated explants at day 0. Insulin-like growth factor (IGF)2, a trigger of trophoblast proliferation (42) and motility (43), was used as positive control. To separately study the effect of IL-33 on proliferative and invasive trophoblast subtypes, we performed invasion assays using growth-arrested primary trophoblasts, which were routinely tested for specific EVT marker expression (Fig. 2B) and cell cycle exit (data not shown). In this study, the invasive capacity of differentiated EVTs was unchanged in the presence of the respective cytokine at a concentration of 10 or 100 ng/ml (Fig. 3B). These data suggest that IL-33 enhances outgrowth of placental explants by preferentially targeting proliferative trophoblast subtypes.

**IL-33 triggers proliferation of first-trimester trophoblasts and placental explants**

To evaluate the anticipated effect of IL-33 on proliferative trophoblasts, we stimulated freshly isolated trophoblasts with rhu IL-33 in the absence or presence of rhu sST2. Of note, these cells display proliferative activity in contrast to long-term (48 h) cultures (data not shown) (37). Evaluation of BrdU incorporation (Fig. 4A) or Ki67 stainings (data not shown) revealed that IL-33 indeed triggers proliferation of trophoblasts in vitro. Moreover, the proliferative effects of IL-33 were comparable with those of IGF2. Although 10 and 100 ng/ml IL-33 showed similar effects on trophoblast proliferation, we used the higher concentration for further experiments, because it was also found to be efficient in placental explant cultures (Fig. 3A). Proliferative activity in the placenta has been ascribed to vCTBs and proximal CCTs, both

![Image](http://www.jimmunol.org/Downloadedfrom/3738_IL-33_TRIGGERS_TROPHOBLAST_PROLIFERATION)

**FIGURE 4.** IL-33 induces proliferation of primary trophoblasts, vCTBs, and CCTs in placental explants. (A) Primary trophoblasts (6–12th week) were pretreated with rhu sST2 (1 µg/ml) for 1 h, followed by incubation with rhu IL-33 (10 or 100 ng/ml) for 18 h. BrdU (10 µM) was added for the last 2 h of culture. IGF2 (100 ng/ml) served as positive control. Five non-overlapping pictures of each condition were taken at a 100-fold magnification, and cells were digitally counted using ImageJ 1.45 software. Proliferation was quantified as the percentage of BrdU+ nuclei of KRT+ trophoblasts relative to the number of DAPI+ nuclei. Bars represent the percentage of the mean (± SD) of four independent experiments (*p < 0.05). Scale bar, 50 µm.
of which express ST2L (Fig. 2A). Consequently, we evaluated BrdU incorporation into these two populations by culturing first-trimester placental floating explants in the absence or presence of rhu IL-33 and/or sST2. BrdU incorporation into hepatocyte growth factor activator inhibitor type 1 (HAI1) vCTBs was significantly increased in the presence of IL-33 (Fig. 4B). Furthermore, proliferation of proximal KRT1 CCTs was markedly increased in IL-33–treated placental explants when compared with control (Fig. 4C). These effects were efficiently blocked by the addition of rhu sST2. We therefore postulate that IL-33 broadly affects trophoblast proliferation by regulating the cell cycle of vCTBs and CCTs.

**IL-33 provokes activation of AKT and ERK1/2**

To define a potential mechanism explaining IL-33–induced proliferation effects on trophoblasts, we assessed whether the phosphorylation pattern of kinases, which have been associated with trophoblast proliferation, was changed in the presence of IL-33. Hence, serum-starved primary trophoblasts were stimulated with rhu IL-33 in the absence or presence of rhu sST2 and subjected to Western blot analyses. Addition of IL-33 induced AKT phosphorylation at amino acid residue Ser473 and phosphorylation of ERK1 and ERK2 at amino acid residues Thr202 and Tyr204, respectively. Notably, addition of sST2 fully inhibited induction of these sites (Fig. 5A). We then tested whether inhibitors targeting upstream activators of AKT or ERK1/2 would interfere with IL-33–induced activation of these signaling kinases. As expected, Western blot analyses of primary trophoblasts demonstrated that inhibitors against PI3K (LY294002) and MEK1/2 (UO126) fully abrogated IL-33–dependent activation of AKT and ERK1/2, respectively (Fig. 5B). To test this observation in a functional assay, we assessed BrdU incorporation into IL-33–treated primary trophoblasts in the absence or presence of LY294002 or UO126. Indeed, both inhibitors abolished IL-33–induced proliferation (Fig. 5C).

**IL-33–induced proliferation of vCTBs and CCTs is mediated via PI3K/AKT and MAPK/ERK signaling**

To identify the specific trophoblast subtypes responsive to IL-33, we stimulated placental explants with rhu IL-33 and analyzed the phosphorylation status of AKT and ERK1/2 in vCTBs and CCTs. Immunofluorescence analyses demonstrated that IL-33 strongly induced phosphorylation of AKT and ERK1/2 in both populations, suggesting an important role for these kinases in IL-33–dependent proliferation (Fig. 6A, 6B). To test this hypothesis, we treated placental explants with LY294002 or UO126 prior to IL-33 stimulation and found that IL-33–induced proliferation of vCTBs and CCTs was fully abrogated in the presence of either of the two inhibitors (Fig. 6C). Notably, treatment with LY294002 or UO126 alone did not alter BrdU incorporation when compared with vehicle-treated control. These data indicate that AKT and ERK1/2 are important mediators of IL-33–induced proliferation in vCTBs and CCTs.

**Discussion**

Successful pregnancy is critically dependent on a homeostatic balance within the highly complex cytokine network during placental development. Besides their important contribution to the maintenance of an anti-inflammatory Th2-biased environment, immunomodulatory factors become increasingly recognized as major regulators of trophoblast function (44, 45). In addition, compromised placental and peripheral cytokine levels are associated with severe pregnancy-related disorders (46, 47). In this study, we demonstrate that the most recently discovered IL-1 family member IL-33 is produced by pMfS and dMfS and regulates the cell cycle of vCTBs and CCTs during the first trimester of pregnancy (Fig. 7B).

Findings from experimental mouse models indicate that extracellular IL-33 is involved in the regulation of various inflammatory

---

**FIGURE 5.** IL-33–mediated activation of AKT and ERK1/2 induces trophoblast proliferation. (A and B) Primary trophoblasts (6–12th week) were serum-starved and pretreated with 1 μg/ml rhu sST2 (A), 10 μM LY294002 (PI3K inhibitor, (B) upper panel), or 10 μM UO126 (MEK1/2 inhibitor, (B) lower panel) for 1 h, followed by stimulation with 100 ng/ml rhu IL-33 for 30 min. Western blot analyses were performed using Abs against phospho (p)-AKT (Ser473), pan-AKT, p-ERK1/2 (Thr202/Tyr204), and pan-ERK1/2. GAPDH served as loading control. One out of three independent experiments is shown in (A) and (B). (C) Primary trophoblasts (6–12th week) were pretreated with LY294002 or UO126 (10 μM each) for 1 h, followed by incubation with rhu IL-33 (100 ng/ml) for 18 h. BrdU (10 μM) was added for the last 2 h of culture. Five nonoverlapping pictures of each condition were taken at a 100-fold magnification, and cells were digitally counted using ImageJ 1.45 software. Proliferation was quantified as the percentage of BrdU+ nuclei of vCTBs and CCTs.
Th1- and Th2-mediated diseases (16, 48, 49) as well as activation of ECs (18, 50). Clinical data show altered IL-33 serum levels in rheumatoid arthritis, ulcerative colitis, or allergic rhinitis (reviewed in Ref. 51). However, surprisingly little is known about active secretion of IL-33 in humans. So far, human fibroblasts have been proposed as the only source for secreted IL-33 (52, 53). Most data available suggest active secretion of IL-33 upon proinflammatory stimuli or mechanical responses in mice (25, 26, 28) or describe its passive release from EC nuclei in the course of necrosis (20). Consequently, tissue damage, for instance, provoked by viral infection (54), is believed to be a prerequisite for nuclear leakage of IL-33. Because tissue damage–associated responses are incompatible with healthy pregnancies, IL-33 secretion from ECs is unlikely to occur in the placenta. Indeed, we found no evidence for secreted IL-33 in culture supernatants of HPAECs or HUVECs. In contrast, pMs and dMs show cytoplasmic IL-33 expression, suggesting that these cells could contribute to extracellular IL-33. Moreover, pMs and dMs express, in addition to full-length IL-33, a shorter form of ∼18–20 kDa (Fig. 1B), which corresponds in size to a recently published secreted IL-33 fragment in mice (21). Interestingly, we were able to demonstrate active secretion of IL-33 from isolated pMs and dMs. Addition of rhu IL-1β further increased IL-33 levels in the culture medium, pointing to paracrine regulation of IL-33 secretion in vivo, as vCTBs were shown to release IL-1β (55). Despite detectable IL-33 serum concentrations in pregnant women, a recent study reports the absence of IL-33 in third-trimester placental explant supernatants or placental perfusates (56). In our study, our findings might provide several explanations for the authors’ unfruitful attempts to verify secreted placenta-derived IL-33. As indicated in Supplemental Fig. 2B, third-trimester placental villi contain only small numbers of macrophages (see also Ref. 57). Therefore, placental IL-33 expression is almost exclusively restricted to the nucleus of ECs, suggesting that no significant secretion of IL-33 takes place at term. In addition, macrophage-derived IL-33 from the underlying mesenchyme of the VC might be immobilized by binding to ST2L-expressing vCTBs. Whether or not placenta-derived IL-33 is secreted into the serum certainly deserves further attention. It is tempting to speculate that dMs might contribute to peripheral IL-33, because these cells appear to maintain IL-33 expression until term (Supplemental Fig. 2B).

Proliferative activity in the human placenta is noticed within the vCTB population covering placental villi and in trophoblasts at the proximal end of the cell column. Continuous division of these cells is integral to proper placental function as both trophoblast subtypes contain progenitor cell populations, which maintain the ST layer or the invasive EVT lineage. However, little is known about the source and nature of signals regulating trophoblast proliferation. Although no data exist to identify a paracrine signal regulating proliferation of CCTs, there is evidence that placental stromal cells secrete IGF1 to trigger vCTB proliferation (42, 58). Interestingly, we detected...
triggers proliferation of these trophoblast subtypes. sST2 acts as a decoy receptor that binds free IL-33 and thereby inhibits IL-33/ST2L signaling.

Interestingly, addition of rhu IL-33 to placental explant cultures elicited a prominent staining for ST2L in proliferative vCTBs and CCTs as well as in invasive EVTs. To assess the effect of IL-33 on trophoblast function, we took advantage of primary in vitro models mimicking trophoblast differentiation. Recently, we (32) and others (36, 37) demonstrated that early cultures of primary first-trimester trophoblasts show a proliferative phenotype similar to vCTBs and CCTs, whereas long-term cultivated trophoblasts undergo growth arrest and acquire a gene expression pattern observed in invasive EVTs in vivo. Western blot and flow cytometric analyses revealed that differentiating trophoblast cultures maintain ST2L expression and display membranous expression of the IL-33R. Interestingly, addition of rhu IL-33 to placental explant cultures provoked enhanced de novo formation of EVTs. To separately study the effect of IL-33 on proliferative and invasive trophoblast subpopulations, we established a novel in vitro invasion model using growth-arrested EVTs. In contrast to its positive effect on EC motility (18, 19), IL-33 did not alter the invasive capacity of EVTs. High sST2 expression levels (Fig. 2B) suggest that invasive EVTs become nonresponsive to exogenous IL-33. EVT-associated sST2 might also function independently of IL-33, as shown by its proinvasive effect on cancer cells (59). Moreover, decidual stromal cells are likely a target of secreted IL-33, because we detected sporadic ST2 expression in these cells (data not shown). To investigate potential proliferation effects of IL-33 on trophoblasts, we first analyzed BrdU incorporation into freshly isolated proliferative EVTs. Although treatment with rhu IL-33 significantly triggered trophoblast proliferation, we were not able to correlate the proproliferative effect to a specific trophoblast subtype, because no specific marker exists to distinguish vCTBs from CCTs in culture. We therefore studied IL-33 effects in placental floating explant cultures, which maintain the anatomical organization of the placenta. This allows the identification of respective trophoblast subtypes according to their spatial distribution (41). In agreement with our studies in primary trophoblast cultures, we found that rhu IL-33 enhances the proliferation rate of vCTBs and CCTs in an ST2L-dependent manner, because its effect was inhibited by the addition of rhu sST2. We propose that IL-33, which we found to be secreted from pMφs, activates vCTBs and CCTs. The latter may also be targeted by dMφ-derived IL-33 being gradually inhibited by induced sST2 expression in growth-arrested invasive EVTs.

IL-33 has been reported to act via NF-kB-, AKT-, and ERK-mediated responses in nonhematopoietic cells (18, 19, 23, 25). Although nuclear translocation of the NF-kB subunit p65 was unaffected upon stimulation with IL-33 (data not shown), the respective cytokine markedly induced phosphorylation of AKT and ERK1/2 in primary trophoblasts. Accordingly, specific inhibitors against PI3K and MEK1/2 blocked IL-33–dependent proliferation. In the next step, we were interested whether vCTBs and CCTs display similar downstream signaling responses to IL-33. Phosphorylation profiles of AKT and ERK1/2 in placental floating explants revealed a clear induction of both kinases in vCTBs as well as in CCTs upon stimulation with IL-33. Finally, inhibition of PI3K or MEK1/2 abrogated IL-33–mediated proliferation in these populations. Therefore, we conclude that IL-33–induced proliferation is mediated via PI3K/AKT and MAPK/ERK signaling. In addition, our data provide for the first time, to our knowledge, information on signaling pathways controlling the cell cycle in CCTs.

In regard to clinics, altered function of macrophages has been linked with PE and intrauterine growth restriction (60). Therefore, our data describing IL-33 expression in pMφs and dMφs add valuable information for the characterization of the yet poorly described phenotype of these cells. By demonstrating IL-33 as the first macrophage-derived factor regulating trophoblast proliferation,
we further strengthen existing data emphasizing the pivotal role of macrophages in placental development (60, 61). Recently, it was reported that IL-33 expression in decidualizing stromal cells is beneficial during the proinflammatory implantation phase, whereas its prolonged expression may cause pregnancy loss (53). In agreement with this study, we showed loss of IL-33 expression in first-trimester decidual stromal cells (Supplemental Fig. 1C). Taken together, these data indicate variability in IL-33 expression and function, ranging from establishment of an autocrine signaling loop in decidual stromal cells during implantation to macrophage-mediated paracrine activation of trophoblasts postplacentation. In summary, we conclude that IL-33/ST2 signaling is a novel pathway to promote trophoblast proliferation and postulate that its deregulation might provoke adverse pregnancy outcome including pregnancy loss and PE.

Acknowledgments

We thank Peter Haslinger, Kristiina Kuusik, and Sarah Machek for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References


13. Hennessy, A., H. L. Pilmore, L. A. Simmons, and D. M. Painter. 1999. A definition of trophoblasts postplacentation. In summary, we conclude that IL-33/ST2 signaling is a novel pathway to promote trophoblast proliferation and postulate that its deregulation might provoke adverse pregnancy outcome including pregnancy loss and PE.

Acknowledgments

We thank Peter Haslinger, Kristiina Kuusik, and Sarah Machek for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References


